0013-7227/06/$15.00/0
Printed in U.S.A.

Endocrinology 147(7):3571–3579

doi: 10.1210/en.2006-0234

Fibroblast Growth Factor-2 Is Expressed by the Bovine Uterus and Stimulates Interferon-τ Production in Bovine Trophectoderm

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Uterine-derived factors are essential for conceptus development and secretion of the maternal recognition-of-pregnancy factor, interferon-τ (IFNT), in ruminant species. The objectives of this study were to determine whether fibroblast growth factor-2 (FGF-2) is expressed in the bovine uterus during early pregnancy in cattle and to determine whether FGF-2 supplementation affects IFNT mRNA and protein abundance in bovine trophectoderm. FGF-2 mRNA was present in endometrium throughout the estrous cycle and was localized to the luminal and glandular endometrial epithelium at d 17–18 after estrus in pregnant and nonpregnant cows. Immunoreactive FGF-2 protein was detected within the endometrium and in the uterine lumen at d 17–18 after estrus, and concentrations did not differ based on pregnancy status. In a bovine trophectoderm cell line, CT-1, supplementation of medium with at least 1 ng/ml FGF-2 increased the incorporation of [^{3}H]thymidine into DNA. Similarly, IFNT secretion from CT-1 cells increased after FGF-2 supplementation (1–100 ng/ml) for 72 h. Abundance of IFNT mRNA in CT-1 cells increased after 24 h exposure to 1, 10, or 100 ng/ml FGF-2. In bovine blastocysts, FGF-2 supplementation did not affect cell number after 72 h of culture but did stimulate IFNT protein concentrations in conditioned medium. In summary, FGF-2 is present in the uterine lumen during early pregnancy and increases IFNT mRNA and protein abundance in trophectoderm. The magnitude by which FGF-2 stimulates IFNT expression suggests that this uterine-derived factor plays an active role in regulating the establishment and maintenance of pregnancy in ruminants. (*Endocrinology* 147: 3571–3579, 2006)

INTERFERON-τ (IFNT) IS the trophectoderm-derived factor that promotes the continuation of pregnancy in cattle, sheep, and other members of the *Ruminantia* suborder of mammals (goats, deer, antelope, and giraffe) (see Refs. 1–3 for reviews). The expression of IFNT is first evident as the trophoblast cell lineage develops at the late morula and early blastocyst stage in cattle (d 6–7 of pregnancy) (4). The production of IFNT mRNA and protein increases profoundly from d 14–21 of pregnancy in the cow and decreases rapidly thereafter coincident with placental attachment to endometrium (5, 6). During the preimplantation period of conceptus development, IFNT interacts with the endometrial epithelium to limit the pulsatile release of prostaglandin $F_{2\alpha}$ and thus prevents corpus luteum regression and return to estrus (7). IFNT also regulates the expression of several uterine-derived factors that potentially function to prepare the uterus for placental attachment, modify the uterine immune system, and regulate early conceptus development (8–13).

First Published Online March 30, 2006

Abbreviations: CIDR, Controlled intravaginal progesterone-releasing device; C_T, threshold cycle; DEPC, diethylpyrocarbonate; DIG, digoxigenin; FAM, 6-carboxyfluorescein; FBS, fetal bovine serum; FGF, fibroblast growth factor; FGFR, FGF receptor; IFNT, interferon-τ; tcRNA, total cellular RNA.

***Endocrinology* is published monthly by The Endocrine Society (http://www.endo-society.org), the foremost professional society serving the endocrine community.**

Uterine secretions are essential for normal conceptus development and the establishment of pregnancy. Defects in conceptus elongation and IFNT production exist in ewes that fail to develop uterine glandular epithelium; a model created by exposing neonatal ewes to progestins (14, 15). Ovine uterine luminal fluids contain known mitogens, such as IGF-I and -II, and other unidentified mitogenic agents during the time of conceptus elongation (16). In addition, several uterine-derived factors have been implicated in directly regulating IFNT production, including granulocyte macrophage-colony stimulating factor (17–19) and IGF-I and -II (16).

Members of the fibroblast growth factor (FGF) family represent a large group of autocrine and paracrine factors that possess various biological activities in a variety of multicellular organisms (20). A subset of the FGF family functions as key regulators of trophectoderm development in certain species. Of particular note is the embryonic action of FGF-4 in mice, where inner cell mass-derived FGF-4 interacts with FGF receptors (FGFR) on trophectoderm (FGFR2) to inhibit the differentiation of mural trophectoderm and thereby provide the conceptus with a proliferating population of placental cells (21, 22). In the pig, FGF-7, or keratinocyte growth factor, is produced by the endometrial epithelium and stimulates trophectoderm proliferation at a critical period of conceptus development (23).

FGF-2, or basic FGF, is a mitogen, morphogen, and an-

giogenic factor that also may play a distinct role during early embryogenesis (24). mRNA for FGF-2 and one of its receptor partners, FGFR2, is present throughout early bovine embryo development, including the blastocyst stage (25, 26). Supplementation with FGF-2 increases the size of mouse trophectoderm outgrowths (27, 28), stimulates gastrulation in rabbit conceptuses (29), and improves bovine embryo development to blastocysts when provided together with TGF-β (30, 31), In addition, endometrial-derived FGF-2 has been implicated in regulating angiogenesis during placental attachment and syncytium formation in the sheep (32). The present study describes work aimed at determining whether FGF-2 is expressed in the bovine endometrium during the estrous cycle and early pregnancy. Also presented here is the first detailed report of FGF-2 acting as a regulator of IFNT expression in bovine blastocysts and a bovine trophectoderm cell line (33).

Materials and Methods

Animal procedures and tissue collection

All animal experimentation was completed in accordance with Institutional Animal Care and Use Guidelines and with the approval of institutional committees for animal care and use at The Pennsylvania State University and the University of Florida. For one experiment, estrus of mature, nonlactating Holstein cows (n = 7) was induced by administering 25 mg Lutalyse (Pharmacia/Upjohn, Kalamazoo, MI) twice im at 14-d intervals. Upon observation of estrus, cows were either artificially inseminated at 12 and 24 h after detecting standing estrus with Holstein semen (Genex Cooperative, Inc., Shawano, WI) or not inseminated upon observation of estrus (nonpregnant). For other studies, follicular development was synchronized in mature Holstein cows by im treatment with 100 μg Cystorelin (Merial Ltd., Iselin, NJ) and insertion of a controlled intravaginal progesterone-releasing device (CIDR) (Pharmacia Animal Health, Kalamazoo, MI) into the vagina. Seven days later, the CIDR was removed, and 25 mg Lutalyse was administered. To ensure that ovulation occurred, 100 μg Cystorelin was provided at 48 h after CIDR removal, and cows were either artificially inseminated at 12 and 24 h after detecting standing estrus with Holstein semen or not inseminated upon observation of estrus.

In one study, endometrial biopsies were collected at d 0 (estrus), 7, 14, and 17 after estrus in nonpregnant cows and d 14 and 17 in pregnant cows (n = 3–5 cows per pregnancy status and day after estrus). An epidural injection of 2% (wt/vol) lidocaine (Sparhawk Laboratories, Inc., Lenexa, KS) was provided to minimize discomfort and limit rectal contractions. Ovaries were palpated to verify that a palpable preovulatory follicle (d 0) or corpus luteum (d 7, 14, and 17) was present on at least one ovary. Uterine biopsies were collected before uterine horns were flushed. Jackson uterine biopsy forceps (4- × 28-mm cutting area; Webster Veterinary Supplies, Inc., Sterling, MA) was inserted through the cervix, and two biopsies were collected approximately halfway between the uterine body and uterotubular junction in the uterine horn ipsilateral to the ovary containing the corpus luteum. Biopsies were examined to ensure that no conceptus tissue was present. Biopsies were snap-frozen in liquid nitrogen and stored at −80 C until use. In cows that were inseminated, both uterine horns were flushed by inserting a latex Foley catheter (Agtech Inc., Manhattan, KS) through the cervix and flushing each horn with 250–500 ml 0.01 M PBS (pH 7.2) containing 0.04% (wt/vol) BSA. Only biopsies derived from cows containing a visible conceptus were included in subsequent work.

In other studies, inseminated and noninseminated cows were euthanized by captive-bolt trauma and exsanguination at d 17–18 after estrus (estrus, d 0). Reproductive tracts were excised, and uterine horns were removed and flushed with 100 ml Dulbecco's PBS (Invitrogen Life Technologies, Carlsbad, CA). For inseminated cows, the presence of a filamentous conceptus in the uterine flush was a prerequisite for sample use in subsequent studies. Conceptus and endometrial tissue was removed from the uterine flush solution by centrifugation (10,000 × *g* for 15 min). Uterine flush solutions were stored at −20 C until use. The uterine horn ipsilateral to the functional corpus luteum was dissected, and endometrial tissue samples were retrieved midway between the uterine-tubular junction and cervix. Samples were snap-frozen in liquid nitrogen and stored at −80 C until use. Cross-sections of the uterine horn ipsilateral to the functional corpus luteum were retrieved, dissected into quarters, and washed in sterile 0.01 M PBS (pH 7.2) that had been pretreated with 0.1% (vol/vol) diethylpyrocarbonate (DEPC). Samples were fixed in 4% (wt/vol) paraformaldehyde (Polysciences, Inc., Warrington, PA) for 8 h on ice and at 4 C overnight in PBS-DEPC containing 10% (wt/vol) sucrose. Tissues were washed in PBS-DEPC, embedded in Tissue-Tek-OCT compound (VWR International, Bridgeport, NJ) during freezing, and stored at −80 C.

In situ hybridization

A 293-bp cDNA that corresponded to the 5′ portion of the coding region for bovine FGF-2 was amplified from bovine endometrial total cellular RNA (tcRNA) by RT-PCR, cloned into the pCR4-Blunt TOPO vector (Invitrogen), and sequenced in both directions to verify the fidelity of the sequence. The cDNA fragment was identical to the sequence previously reported for bovine FGF-2 (GenBank accession no. M13440). Sense and antisense templates were generated by PCR using primers that contained a T7 RNA polymerase binding site. Riboprobes were synthesized using a digoxigenin (DIG) RNA labeling kit (Roche Molecular Biochemicals, Indianapolis, IN).

Uterine endometrium cryosections (10 μm) were dried on Superfrost-plus slides (VWR International, West Chester, PA). Sections were washed with 10 mM Tris (pH 8.0) containing 1 mM EDTA (pH 8.0) for 10 min and postfixed with 4% (wt/vol) paraformaldehyde for 10 min. Sections were prehybridized using DIG RNA hybridization buffer at 50 C for 2 h. DIG-labeled sense or antisense FGF-2 riboprobes were denatured at 95 C for 5 min and added to the hybridization buffer at a final concentration of 200 ng RNA/ml. Sections were incubated with riboprobes for 16 h at 50 C in a humidified environment and were washed with graded concentrations of sodium chloride/sodium citrate solution (2× and 0.2×) at 50 C. Sections were incubated with alkaline phosphatase-conjugated anti-DIG antibody for 1 h at room temperature and developed with nitroblue tetrazolium/5-bromo-4-chloro-3-indolyl phosphate according to the manufacturer's specifications (Roche Molecular Biochemicals). Slides were mounted and visualized under bright-field microscopy at a 200-fold magnification. Multiple sections from each endometrial sample were probed and analyzed (n = 12 sections per cow).

FGF-2 ELISA

Endometrial samples from cows killed at d 17–18 after estrus were homogenized in lysis buffer [50 mM Tris-HCl (pH 7.0), 50 mM NaCl, 1 mM EDTA, 1 mM 4-(2-aminoethyl)benzenesulfonyl fluoride, 10 μM E64] and centrifuged (10,000 × *g*) to remove particulate matter. Uterine flush samples were concentrated using Centriprep concentrators (3000 MWCO; Millipore Corp., Bedford, MA). Amount of total protein was determined by using the Coomassie-Plus Protein Assay (Pierce Corp., Rockford, IL). Samples were stored at −20 C until use.

A human FGF-2 ELISA kit (EMD Biosciences, San Diego, CA) was used to quantify FGF-2 protein levels from bovine uterine flushes and endometrial lysates (sensitivity = 0.025 pg/ml FGF-2). Bovine FGF-2 is 98.7% identical in amino acid sequence with human FGF-2, and this human-based ELISA exhibits 100% cross-reactivity with bovine FGF-2. This ELISA has limited cross-reactivity with bovine acidic FGF (FGF-1) (0.15% cross-reactivity, 1.15% interference) and human FGF-4 (0.02% cross-reactivity, 0.2% interference) and no cross-reactivity with other FGFs. Both human and bovine FGF-2 proteins (R&D Systems, Inc., Minneapolis, MN) were included as standards. Flush solution (Dulbecco's PBS) was included as a negative control. Results obtained were extrapolated to adjust for total protein concentration of samples and original uterine flush volume.

Trophectoderm cell culture

The bovine CT-1 cell line developed by Talbot *et al.* (33) initially was propagated in a coculture system using mitotically inactivated STO cells as a feeder system (33). To limit the possible confounding effects of the

feeder cells, a feeder-independent culture system was developed by passaging cells onto culture-ware treated with Matrigel basement membrane matrix (BD Biosciences, Bedford, MA). After two passages, residual STO feeder cells could no longer be detected microscopically. Cells have since been propagated through 50+ passages on Matrigel-coated plates and continue to grow as a monolayer in distinct colonies and retain characteristic features of trophectoderm cells (small cuboidal shape, prominent nuclei, numerous secretory granules, and dome formation within the cell monolayer).

After the feeder cell-independent cell system was established, CT-1 cells were propagated at 38.5 C (5% CO_2 in air) in DMEM containing 10% (vol/vol) fetal bovine serum (FBS) (Invitrogen) and other supplements (4.5 g/liter D-glucose, nonessential amino acids, 2 mM glutamine, 2 mM sodium pyruvate, 55 μM β-mercaptoethanol, 100 U/ml penicillin G, 100 μg/ml streptomycin sulfate, and 250 ng/ml amphoterin B). Because trypsin and other enzymatic cell disruption reagents damage CT-1 cells, the cells were passaged by manually scraping them from the plates and passing them through a small-bore needle to generate small clumps of cells. Cells were serum starved for 24 h before and throughout experiments by incubation in serum-free DMEM containing all other supplements listed above and 10 μg/ml insulin, 5.5 μg/ml transferrin, and 6.7 ng/ml sodium selenite (Invitrogen).

In vitro production and culture of bovine embryos

Bovine embryos were produced by *in vitro* maturation, fertilization, and culture procedures described elsewhere (34). At d 8 after fertilization, expanded and hatched blastocysts were placed in groups of eight to 11 in 50-μl drops of G2 medium containing BSA (Vitrolife, Inc., Englewood, CO), 5% FBS, and 0, 1, or 100 ng/ml FGF-2 (R&D Systems). After 72 h, embryos were retrieved and fixed in 4% paraformaldehyde in PBS (Polysciences). Conditioned medium from each culture drop was collected and stored at −20 C until antiviral activity was determined. Cells per blastocyst were counted by staining the blastocysts with Hoechst 33342 (10 μg/ml in PBS), cutting open each blastocyst with 27-gauge hypodermic needles, squashing it on a glass slide with a coverslip, and viewing the cells' nuclei by fluorescent microscopy.

[^{3}H]Thymidine incorporation analysis

CT-1 cells were seeded onto Matrigel-coated 24-well plates at a low density (~50 cells/mm^2) in DMEM containing FBS and other additives. This low density of cell seeding was completed to minimize the confounding effects of contact inhibition on the outcome of the studies. After cells were allowed to attach to the matrix for 48 h, medium was removed and replaced with medium lacking FBS. After 24 h of serum starvation, fresh serum-free medium containing 0, 1, 10, or 100 ng/ml FGF-2 (R&D Systems) and 0.5 μCi/ml [^{3}H]thymidine (50 Ci/mmol) (MP Biomedicals, Irvine, CA) was added to cultures (four replicate wells per treatment, three independent studies). Cell confluency was estimated to be between 5 and 10% at the time FGF-2 supplementation began. Twenty-four hours later, the cells were washed with 0.01 M PBS, and DNA was precipitated with ice-cold 5% (wt/vol) trichloroacetic acid for 20 min. After washing with 70% (vol/vol) ethanol and PBS, DNA was solubilized in 0.5 N NaOH and 1% (vol/vol) Triton X-100. Scintillation cocktail (Fisher Scientific, Fair Lawn, NJ) was added and samples were analyzed using liquid scintillation counting.

Analysis of IFNT secretion

CT-1 cells were seeded at a normal density (~250 cells/mm^2) onto Matrigel-coated 24-well plates and incubated in DMEM containing FBS and other additives for 5 d. Subsequently, medium was removed and replaced with DMEM lacking FBS. Twenty-four hours later, fresh serum-free medium containing 0, 0.01, 0.1, 1, 10, or 100 ng/ml FGF-2 was added to cultures (four replicate wells per treatment, four independent studies). At 72 h after treatment, medium was harvested and stored at −20 C. Viable cell number was determined by using the Cell Titer 96 Aqueous One Solution Cell Proliferation Assay (Promega Corp., Madison, WI). Absorbance was measured at 490 nm.

The quantity of biologically active IFNT in CT-1 cell-derived medium was determined by antiviral assay (35). The ability of samples to prevent vesicular stomatitis virus-induced cell lysis by 50% was compared with a recombinant human IFN-α standard (EMD Biosciences) (3.84×10^8 IU/mg). Data are expressed as international units per milliliter of conditioned medium at the end of the treatment period (72 h for both CT-1 cells and blastocysts). This assay is unable to discriminate between the various IFNs that may exist within a biological sample. This is of little consequence in the present studies because IFNT is the predominant IFN produced (>99%) by bovine conceptuses (36). The antiviral assay is extremely sensitive, and this sensitivity is required for detecting IFNT in blastocyst-conditioned medium (4).

Western blotting

CT-1 conditioned medium (200 μl) collected from cells exposed to 0 or 100 ng/ml FGF-2 (R&D Systems) for 72 h (n = 2 wells per treatment) was acetone precipitated, resolubilized, heat denatured, and loaded onto a 15% (wt/vol) polyacrylamide gel. Samples were electrophoresed under denaturing conditions (37). After SDS-PAGE, proteins were blotted onto nitrocellulose membranes, blocked with 5% (wt/vol) nonfat dry milk, and incubated with bovine IFNT antiserum (1:2000) (generously provided by Dr. R. M. Roberts, University of Missouri). Immunoreactivity was determined by incubation in secondary antibody (goat anti-rabbit IgG conjugated with horseradish peroxidase) and development using enhanced chemiluminescence (GE Healthcare Bio-Sciences Corp., Piscataway, NJ). Recombinant bovine IFNT1c protein was included as a positive control (5).

Quantitative RT-PCR

Quantitative real-time RT-PCR was used to measure the relative abundance of FGF-2 mRNA in endometrium derived from pregnant and nonpregnant cows and IFNT mRNA in FGF-2-treated CT-1 cells. The RNAqueous Midi RNA isolation kit (Ambion, Inc., Austin, TX) was used to extract tcRNA from bovine endometrial samples. CT-1 cells (~250 cells/mm^2) were seeded onto Matrigel-coated six-well plates and incubated in DMEM containing FBS and other additives for 5 d. Medium then was removed and replaced with medium lacking FBS. After 24 h, fresh serum-free medium containing 0, 1, 10, or 100 ng/ml FGF-2 was added to cultures. Extraction of tcRNA was performed at 24 h after treatment using the RNAqueous-4 PCR kit (Ambion) (n = 2 replicate wells per treatment; four independent studies).

All samples were incubated with RNase-free DNase (Ambion) at the end of RNA extraction and again immediately before RT. The High Capacity cDNA Archive Kit and random hexamers (Applied Biosystems, Foster City, CA) was used for RT of tcRNA (20 ng for IFNT analysis, 100 ng for FGF-2 analysis). Specific primers and probe sets were used for amplifying RT product from endometrial samples and CT-1 cells (Table 1). The IFNT primers and probe were developed to recognize every known bovine and ovine IFNT isoform. The FGF-2 and IFNT probes were labeled with fluorescent 5′ reporter dye 6-carboxyfluorescein (FAM) and 3′ quencher (BHQ-1) (Biosearch Technologies, Novato, CA). Forty cycles of PCR were completed using the TaqMan Universal PCR Master Mix (Applied Biosystems) and the 7300 Real-Time PCR System (Applied Biosystems). Abundance of 18S RNA was used as a loading control by adding primers and an 18S probe containing a VIC-labeled 5′ fluorescent reporter and 3′ 6-carboxy-tetramethylrhodamine quencher (18S RNA Control Reagent Kit; Applied Biosystems) within the PCR. Each RNA sample was analyzed in triplicate reactions. A fourth

TABLE 1. Bovine IFNT and FGF-2 primers and probe sequences used for quantitative real-time RT-PCR

Primer/probe	Sequence (5′–3′)
IFNT SE	TGCAGGACAGAAAAGACTTTGGT
IFNT AS	CCTGATCCTTCTGGAGCTGG
IFNT probe	TTCCTCAGGAGATGGTGGAGGGCA
FGF-2 SE	ACCGGTCAAGGAAATACTCCAG
FGF-2 AS	CAGGTCCTGTTTTGGGTCCA
FGF-2 probe	TGGTATGTGGCACTGAAACGAACTGGG

Probes were synthesized with a FAM reporter dye and BHQ-1 quencher. AS, Antisense primer (3′ primer); SE, sense primer (5′ primer).

reaction lacking exposure to the reverse transcriptase was included for each sample to verify they were free of genomic DNA contamination.

The comparative threshold cycle (C_T) method was used to quantify the abundance of FGF-2 and bovine IFNT mRNA relative to that of 18S RNA (ABI Prism Sequence Detection System User Bulletin No. 2; Applied Biosystems). The C_T number for FAM (FGF-2 or IFNT mRNA) and VIC (18S RNA) fluorescence was calculated within the geometric region of the plot generated during PCR. The ΔC_T value was determined by subtracting the 18S C_T value from the FGF-2 or bovine IFNT C_T value of the same sample. The $\Delta\Delta C_T$ for each sample was calculated by subtracting the highest sample ΔC_T value (*i.e.* the sample with the lowest target expression) with the remaining values. Because each unit of $\Delta\Delta C_T$ difference is equivalent to a doubling in the amplified PCR product, fold changes in relative FGF-2 or bovine IFNT mRNA abundance was determined by solving $2^{-\Delta\Delta CT}$.

Statistical analyses

All analyses were completed by least-squares ANOVA using the general linear model of the Statistical Analysis System (SAS Institute Inc., Cary, NC). Differences between individual means were partitioned further by completing pairwise comparisons [PDIFF (probability of difference) analysis in SAS]. When analyzing real-time RT-PCR results, the ΔC_T values were used for the analyses, but data are presented as fold differences from the lowest expression value. Antiviral activity of CT-1 conditioned medium was log-transformed and normalized based on cell number before analysis. Results are presented as arithmetic means ± SEM.

Results

FGF-2 is expressed in the bovine endometrium and is present in the uterine lumen during diestrus

Quantitative real-time RT-PCR was completed on tcRNA isolated from uterine biopsies collected throughout the estrous cycle to determine whether FGF-2 mRNA is present in the bovine endometrium (Fig. 1). FGF-2 mRNA was detected throughout the estrous cycle. Quantities of FGF-2 mRNA relative to that of an internal control (18S RNA) were greater ($P = 0.01$) in endometrium collected on the day of estrus (d 0) than in endometrium collected at d 7, 14, and 17 after estrus. Abundance of endometrial FGF-2 mRNA was not influenced significantly by pregnancy status at d 14 and 17 after estrus.

An FGF-2 ELISA was used to determine concentrations of FGF-2 protein in endometrium and uterine flushes derived from pregnant and nonpregnant cows at d 17–18 after estrus (Table 2). Endometrial FGF-2 concentrations were not different statistically between groups. Similarly, concentrations of FGF-2 protein in uterine flushes did not differ based on pregnancy status regardless of whether data were evaluated based on total protein of uterine flushes or on total amount of FGF-2 in each flush.

In situ hybridization was completed to localize FGF-2 mRNA within the bovine endometrium of pregnant and nonpregnant cows at d 17–18 after estrus (Fig. 2). Intense staining for FGF-2 mRNA was observed in the luminal epithelium of pregnant and nonpregnant cows. More subtle staining also was evident within shallow and deep glandular epithelium. The intensity of glandular epithelial staining was less than that of the luminal epithelium in most of the sections. However, there were no discernable differences in the intensity of FGF-2 mRNA staining in the luminal and glandular epithelium between pregnant and nonpregnant cows. Slight stromal staining that was noticeably different from sense control sections was observed in some but not all cows. Several sections derived from pregnant cattle, including the one presented in Fig. 2, exhibited a partial loss of endometrial epithelium. This is consistent with the beginning of syncytium formation, which results from the invasion of binucleated cells from the trophectoderm into the uterine epithelium (38).

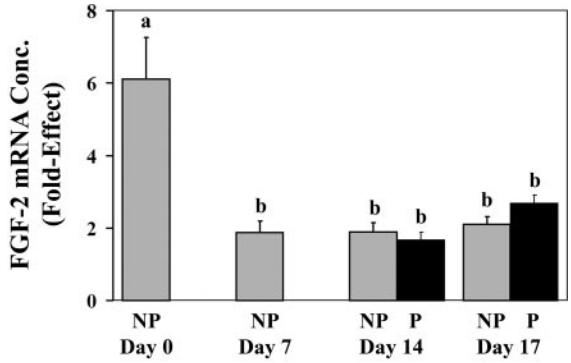

FIG. 1. Relative abundance of FGF-2 mRNA in bovine endometrium throughout the estrus cycle and early pregnancy. Quantitative real-time RT-PCR was used to determine the abundance of FGF-2 mRNA in tcRNA derived from endometrium of cyclic cows at d 0 (estrus), 7, 14 and 17 after estrus (nonpregnant; NP) and from d 14 and 17 after breeding (pregnant; P) (n = 3–5 cows/time point and pregnancy status). Abundance of 18S RNA was used as an internal control to normalize FGF-2 values. ΔC_T values were used to analyze the data and data are presented as mean fold-differences ± SEM relative to the lowest expression value. *Different superscripts over data bars* represent differences ($P = 0.01$) among time points and pregnancy status.

Supplementation with FGF-2 increases the rate of DNA synthesis in CT-1 cells

CT-1 cells were seeded onto Matrigel-coated 24-well plates at a low density to measure the rate of DNA synthesis of these cells in response to FGF-2. Supplementing CT-1 cells with 1 ng/ml FGF-2 for 24 h increased ($P < 0.05$) the rate of [^{3}H]thymidine incorporation into DNA when compared with untreated controls (Fig. 3). Greater doses of FGF-2 (10 or 100 ng/ml) did not further increase the rate of [^{3}H]thymidine incorporation. This lack of a dose response implies that 1

TABLE 2. Quantities of immunoreactive FGF-2 in the uterine lumen and endometrium of d 17–18 pregnant and nonpregnant cows

Pregnancy status	Uterine luminal FGF-2 (pg FGF-2/mg total protein)	Uterine luminal FGF-2 (ng FGF-2/flush)	Endometrial FGF-2 (ng FGF-2/mg total protein)
Nonpregnant	70.31 ± 20.76	7.59 ± 1.53	2.46 ± 0.28
Pregnant	102.00 ± 22.61	11.85 ± 4.92	2.66 ± 0.56

Data represent means and SEM of FGF-2 concentrations from uterine luminal flushes (concentration based on amount of total uterine luminal protein and total amount of FGF-2 in the uterine flush) and from endometrial lysates (n = 3–7 cows per pregnancy status). No significant differences were detected between pregnant and nonpregnant cows.

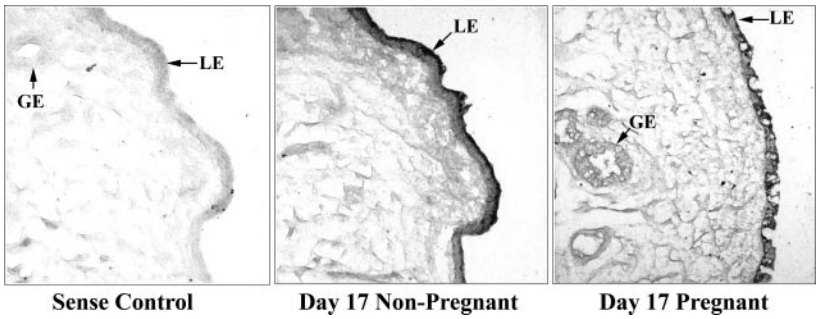

FIG. 2. Localization of FGF-2 mRNA in the bovine endometrium at d 17 after estrus. In-situ hybridization using DIG-labeled sense (negative control) and antisense FGF-2 riboprobes showed that FGF-2 mRNA localized to the luminal (LE) and glandular (GE) epithelium in pregnant and nonpregnant cows at d 17 after estrus. Slight staining of stromal endometrium that was greater than that of the negative controls was observed in some but not all sections. Representative sections at ×200 magnification are depicted.

ng/ml FGF-2 was sufficient to maximally stimulate CT-1 DNA synthesis response.

Supplementation with FGF-2 increases IFNT mRNA and protein concentrations in CT-1 cells

Bovine FGF-2 was provided at various concentrations to CT-1 cells to determine whether FGF-2 supplementation increases the rate of IFNT protein production in bovine trophectoderm. Medium was collected after 72 h of exposure to FGF-2, and IFNT concentrations in conditioned medium were measured with antiviral assays. Antiviral results were normalized by CT-1 cell number at the end of the incubation period. The FGF-2 preparation used for this and other studies did not contain antiviral activity. FGF-2 supplementation induced a dose-dependent increase in IFNT concentrations (Fig. 4). The first significant increase ($P < 0.01$) in IFNT concentrations occurred when supplementing with 0.1 ng/ml FGF-2 (1.5-fold increase over controls). Treatment with 10 or 100 ng/ml FGF-2 induced a 4.6- and 7.6-fold increase in IFNT concentrations, respectively, when compared with controls.

Although the antiviral assay system provides a sensitive means of quantifying IFNs in biological samples, it is unable to discriminate between various type I and type II IFNs within samples. Western blot analysis was completed to verify that IFNT concentrations in CT-1 cell conditioned medium were influenced by FGF-2 supplementation (Fig. 5). When using an IFNT-specific polyclonal antiserum (39), immunoreactive IFNT bands were greater in intensity in medium collected from CT-1 cells supplemented with 100 ng/ml FGF-2 for 72 h than from cells not exposed to FGF-2. The apparent absence of immunoreactive IFNT in untreated samples does not indicate that CT-1 cells do not normally produce IFNT. Immunoreactive protein bands could be detected in these samples when the blot was exposed to film for longer periods of time (data not shown). Band sizes ranged from 22–26 kDa, which correspond with the sizes of glycosylated IFNT isoforms detected from bovine conceptus secretions (40, 41). The recombinant bovine IFNT control (19 kDa) is not glycosylated and therefore is smaller in size. No nonspecific protein bands were evident on a companion blot reacted only with the secondary antibody (data not shown).

Quantitative real-time RT-PCR was completed to determine whether FGF-2 regulates the abundance of IFNT mRNA in CT-1 cells (Fig. 6). A dose-dependent response in IFNT mRNA abundance was observed after 24 h of FGF-2 supplementation. The lowest concentration of FGF-2 tested (1 ng/ml) was sufficient to increase ($P < 0.01$) the relative abundance of IFNT mRNA over that of the control. Additional increases in IFNT mRNA abundance were evident when 10 or 100 ng/ml FGF-2 was supplemented to the culture medium (6.30- and 13.2-fold increase, respectively; $P < 0.01$).

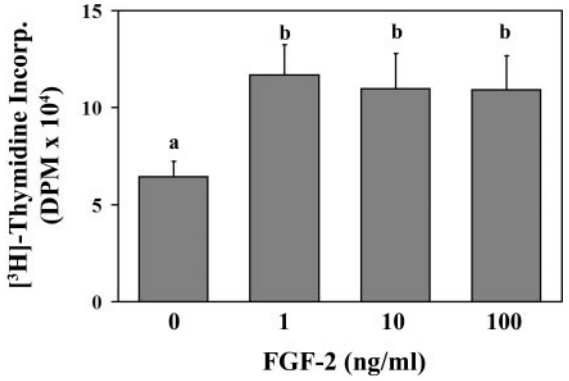

FIG. 3. The effect of FGF-2 supplementation to medium on [^{3}H]thymidine incorporation into CT-1 cell DNA. Cells were exposed to 0.5 μCi/ml [^{3}H]Thymidine for 24 h in the presence of 0, 1, 10, or 100 ng/ml FGF-2 (n = 4 wells/treatment; three independent studies). Precipitated DNA was analyzed by liquid scintillation counting. *Different superscripts over data bars* represent statistically significant differences between treatments ($P < 0.05$).

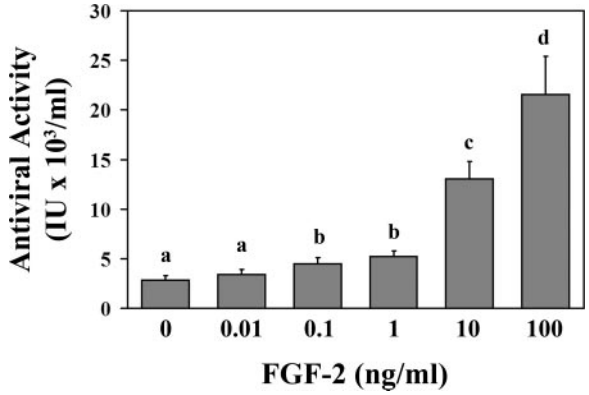

FIG. 4. Increase in antiviral activity CT-1 cell conditioned medium in response to FGF-2 supplementation. Cells were incubated in medium containing 0, 0.01, 0.1, 1, 10, or 100 ng/ml FGF-2 for 72 h (n = 4 wells/treatment; four independent studies). Antiviral activity of conditioned medium is expressed as international units (IU) of antiviral activity per ml of medium. Viable CT-1 cell number was determined by quantifying the amount of tetrazolium (MTS) oxidation (measured by absorbance reading at 490 nm) and antiviral activity results were adjusted accordingly. *Different superscripts over data bars* represent statistically significant differences among treatments ($P < 0.01$).

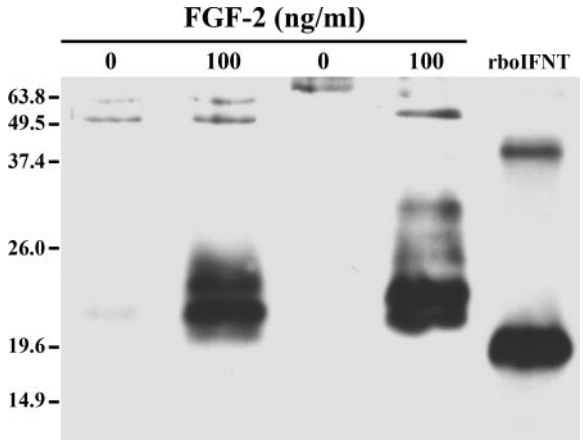

FIG. 5. Western blot demonstrating increased IFNT concentrations in CT-1 cell conditioned medium in response to FGF-2 supplementation. Cells were incubated in the presence or absence of 100 ng/ml FGF-2 for 72 h. Immunoreactive IFNT protein bands ranged from 22 to 26 kDa in size. A recombinant bovine IFN-τ protein (boIFN-τ1c; 19 kDa) was included as a positive control.

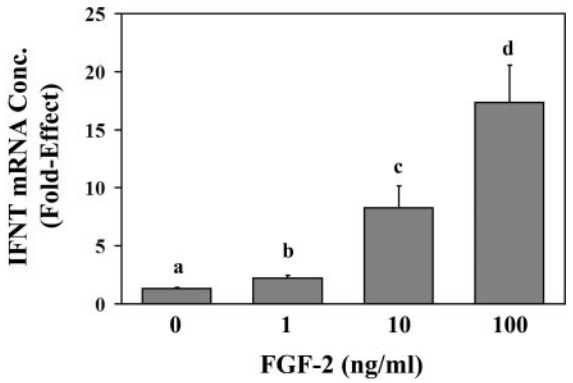

FIG. 6. Increase in the relative abundance of IFNT mRNA in response to FGF-2 treatment of CT-1 cells. CT-1 cells were incubated in medium containing 0, 1, 10 or 100 ng/ml FGF-2 for 24 h. TcRNA was extracted and relative abundance of IFNT mRNA was determined by quantitative real-time RT-PCR. Abundance of 18S RNA was used to normalize IFNT values. ΔC_T values were used to analyze the data (n = 2 wells/treatment; four independent studies) and data are presented as mean fold-differences ± SEM from the lowest expression value. *Different superscripts over data bars* represent statistically significant differences between treatments ($P < 0.01$).

Supplementation with FGF-2 increases the production of IFNT in bovine blastocysts

In vitro-generated bovine blastocysts were used to evaluate whether FGF-2 actions also were evident in preimplantation bovine embryos. Groups of blastocysts at d 8 after fertilization were allocated to medium drops (n = 8–11 embryos per medium drop) supplemented with 0, 1, or 100 ng/ml FGF-2. After 72 h of incubation (d 11 after fertilization), a dose-dependent increase ($P < 0.05$) in antiviral activity of conditioned medium was detected when providing 1 and 100 ng/ml FGF-2 (Fig. 7A). Total number of cells per embryo did not differ after the 72-h incubation with FGF-2 (Fig. 7B). A dose-response effect of FGF-2 supplementation on antiviral activity in conditioned medium remained evident after results were normalized based on the total embryo cell number within each medium drop (data not shown).

Discussion

One of the primary causes of embryonic mortality in cattle and other ruminants is failure of the conceptus to develop an adequate trophectoderm and produce a sufficient amount of IFNT (42, 43). Many of the key regulatory factors controlling conceptus development in ruminants remain undefined, but conceptus survival undoubtedly depends on signals originating from both the embryo and uterus. The present study made a key discovery in describing FGF-2 as a mediator of IFNT expression. This study initially was completed by using the CT-1 cell line. This cell line was derived from an outgrowth of trophectoderm originating from a single bovine blastocyst (33). CT-1 cells are similar morphologically to *in vivo* trophectoderm cells and produce IFNT constitutively at a level comparable to blastocyst-stage bovine embryos. The latter characteristic underscores their value as a model system for screening putative IFNT regulatory factors.

The IFNT secretory response to FGF-2 supplementation was consistent between CT-1 cells and bovine blastocysts. Based on results derived from CT-1 cells, this action is mediated in large part at the level of gene transcription and/or IFNT transcript stability. The mechanisms used by FGF-2 to regulate IFNT gene expression have not been uncovered. Cytoplasmic domains of FGFR contain intrinsic tyrosine kinase activity that regulates numerous signaling pathways, including protein kinase C and Ras-mediated systems such as the MAPK pathway (44). Both these signaling systems regulate IFNT gene transcription. Treatment of human choriocarcinoma (JEG3) cells with a protein kinase C activator (phorbol-12-myristate-13-acetate) increases activator protein 1-driven IFNT reporter gene transcription (45, 46). Ras-mediated signaling systems are known to enhance Ets-2 activity, and phosphorylation of this transcription regulator is required for maximal IFNT promoter/enhancer activity in murine 3T3 fibroblasts (47, 48).

Results from these studies argue that FGF-2 is a weak mitogen but a strong IFNT regulatory factor in bovine trophectoderm. A subtle increase in DNA synthesis was detected by providing 1 ng/ml FGF-2 to CT-1 cells, and no mitogenic response was detected in bovine blastocysts. Actual CT-1 cell counts could not be completed, at least not accurately, because cells could not be separated from one another after enzyme treatment (data not shown). Therefore, it remains uncertain whether the increase in CT-1 DNA synthesis reflects stimulation in rate of mitosis or whether the outcome resulted from FGF-2 stimulating progression through the S-phase of the cell cycle without cytokinesis. The latter possibility may explain why greater quantities of FGF-2 were unable to further stimulate the rate of DNA synthesis. The blastocyst study was not designed to detect weak proliferative effects of FGF-2. Embryos were cultured in groups and were incubated in medium containing FBS. This serum contained small amounts of FGF-2 (50–70 pg/ml according to the manufacturer). Also, current embryo culture systems do not adequately mimic the *in utero* environment to support growth past the hatching blastocyst stage. Any of these factors could have prevented the detection of a slight mitogenic response to supplemental FGF-2. By contrast, dramatic ef-

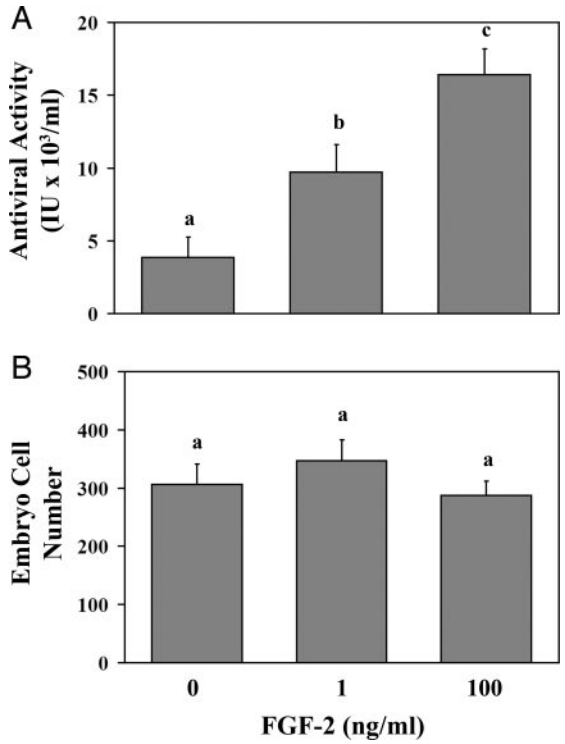

FIG. 7. The effect of FGF-2 medium supplementation on antiviral activity of conditioned medium and cell number of *in vitro* produced bovine blastocysts. *In vitro*-derived bovine blastocysts were collected at d 8 after fertilization and placed in groups (n = 8–11) into 50-μl drops of medium containing 0, 1, or 100 ng/ml FGF-2 (four independent studies). The studies were terminated after 72 h of incubation with treatments. A, Antiviral activity (means ± SEM) of conditioned embryo medium (expressed as IU/ml) after FGF-2 supplementation. B, The effect of FGF-2 supplementation on embryo cell number at d 11 after fertilization (means ± SEM). *Different superscripts over data bars* represent statistically significant differences between treatments ($P < 0.05$).

fects on IFNT mRNA and protein concentrations are evident with FGF-2 supplementation. It is intriguing that the FGF-2 concentration generating the most profound effect of IFNT expression (100 ng/ml) is so much greater than the FGF-2 dose needed to produce an effect on DNA synthesis (1 ng/ml). The reason for this disparity has not been examined, but it is conceivable that these two activities are mediated through different FGFR subtypes. Four distinct genes encode FGFR, and three of these gene products generate spliced variant receptor forms (49). FGF-2 is known to interact with FGFR1 (IIIb and IIIc spliced variant forms), FGFR2-IIIc, FGFR3-IIIc, and FGFR-4 with different affinities (49). Therefore, the fundamental regulator of IFNT expression may lie with the activity of a specific FGFR subtype.

Concentrations of endometrial FGF-2 mRNA were constant throughout the progesterone-dominated phase of the cycle, but a greater level of FGF-2 mRNA was detected at estrus. Estrogens stimulate FGF-2 expression in human endometrial cancer cell lines (50) and in endometrium from ovariectomized ewes (51) and rats (52), whereas progesterone diminishes estrogen-stimulated FGF-2 production in the rat uterus (52). However, progesterone alone increases endometrial FGF-2 mRNA in rodents (52). The steroid dependence of uterine FGF-2, and particularly its responsiveness to estrogens, has been implicated in regulating angiogenesis, hyperemia, and other aspects of tissue remodeling that occur during estrogen-dominated phases of estrous or menstrual cycles in humans, sheep, and rodents (53). In the present study, estradiol and progesterone concentrations were not quantified, but our assessment of ovarian structures at the time of biopsy collection support the concept that elevated estrogen concentrations during estrus (presence of a preovulatory follicle, absence of a corpus luteum) increases FGF-2 expression in bovine endometrium when compared with expression levels during the luteal phase of the estrous cycle.

FGF-2 mRNA was localized primarily to the luminal epithelium at d 17 after estrus in cows. Less intense staining for FGF-2 mRNA was detected in the glandular epithelium, and very little staining was evident in the stroma. No attempts were made to determine whether differences in FGF-2 mRNA abundance exist between caruncular and intercaruncular regions of the uterus. Pregnancy status did not affect FGF-2 mRNA and protein concentrations in the bovine uterus. These observations are distinct from that of other species. In porcine endometrium, FGF-2 protein is localized primarily to the luminal epithelium and less so to the glandular epithelium from d 12–14 of pregnancy, but little immunoreactive FGF-2 exists in luminal and glandular epithelium in nonpregnant gilts (54). In the rabbit, FGF-2 protein is abundant in luminal epithelium and absent in glandular epithelium in pregnant and pseudopregnant states (55). In the rat uterus, FGF-2 protein localizes to the luminal and glandular epithelium throughout diestrus and is present in uterine luminal flushes at d 4–5 of pregnancy but not before (56). Such divergence in uterine expression patterns for FGF-2 raise the possibility that species-specific roles for FGF-2 exist during early pregnancy.

The bovine endometrium is a likely source of FGF-2 for the conceptus during early pregnancy but is not necessarily the only source of FGF-2 within the uterus. FGF-2 mRNA is present in bovine conceptuses throughout early development (25, 26). The present study did not detect a conceptus contribution to FGF-2 in the uterine lumen. This is not surprising because FGF-2 protein usually is sequestered locally within extracellular matrices, commonly bound to heparin or heparin sulfate (57), and conceptus-derived FGF-2 may have been localized to an embryonic compartment that was not exposed to the flush solution (54). Detecting FGF-2 in uterine luminal flushes suggests that the endometrial epithelium is providing FGF-2 to the developing conceptus. Extreme care was taken when flushing uteri to ensure that the integrity of the endometrium was not compromised (*i.e.* luminal and glandular epithelium were not sheared). Also, cellular debris was readily removed from the flush before freezing the samples. In all likelihood, the gentle flushing procedure was not rigorous enough to remove all the extracellular-associated FGF-2 from the uterine lining, and the assessment of uterine FGF-2 concentrations probably is less than their actual value.

In conclusion, FGF-2 is expressed by the endometrium and present within the uterine lumen during early pregnancy in cattle. Moreover, FGF-2 is, at best, a weak mediator of trophectoderm proliferation but is a strong regulator of IFNT

production in bovine trophectoderm cells and blastocyst-stage bovine embryos. Collectively, these findings provide new insight into how the bovine conceptus develops and communicates its presence in the uterus during early pregnancy.

Acknowledgments

We thank Dr. R. Michael Roberts (University of Missouri) for generously donating the bovine IFNT antiserum and Mr. Glen Gilbert and Mr. Michael Kaproth of the Genex Cooperative, Inc., for donating Holstein semen.

Received February 22, 2006. Accepted March 22, 2006.

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This work was supported by National Research Initiative Competitive Grants 2003-35203-13345 (to A.D.E.) and 2003-35203-15382 from the United States Department of Agriculture Cooperative State Research, Education, and Extension Service.

Author disclosure summary: D.D.M., I.M.A, O.M.O., A.M.P., N.C.T., S.E.J., and A.D.E. have nothing to declare.

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